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<b>(54) Title:</b> MODULATOR OF INFLAMMATION  <div style="text-align: center;"><b>5'-ACAGACCACAGACC</b></div> <b>(57) Abstract</b> <p>Two novel DNA binding proteins are provided which bind specifically to DNA target sites within the promoter region of the TNF-<math>\alpha</math> gene. Also provided are screening methods based on this DNA-protein interaction which may be used to identify compounds which modulate the expression of TNF-<math>\alpha</math> and a genetic screen for susceptibility to inflammatory disease based upon screening for a single nucleotide polymorphism in the TNF-<math>\alpha</math> promoter.</p>		

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MODULATOR OF INFLAMMATION

The present invention is concerned with a specific molecular mechanism by which inflammation and susceptibility to infectious disease may be genetically regulated, more specifically with a molecular mechanism modulating the expression of tumour necrosis factor- $\alpha$  (TNF- $\alpha$ ). In particular, the invention relates to two novel DNA binding proteins which bind specifically to DNA target sites in the TNF- $\alpha$  promoter region, to assays based on this protein/DNA interaction and to a method of screening for susceptibility to inflammatory disease based upon screening for a polymorphism in the TNF- $\alpha$  promoter.

Tumour necrosis factor- $\alpha$  is a pro-inflammatory cytokine believed to play an important role in the pathogenesis of many inflammatory and severe infectious diseases. An example of a disease whose severity is known to be associated with TNF- $\alpha$  is fatal cerebral malaria which occurs in a small proportion of individuals infected with the malaria parasite *Plasmodium falciparum*. Clinical studies of cerebral malaria patients have demonstrated an association between host TNF- $\alpha$  levels and disease severity (Grau, G. E. et al. (1989) *New Engl. J. Med.* **320**: 1586-1591; Kwiatkowski, D. et al (1990) *Lancet* **336**: 1201-1204; Kern P. et al. (1989) *Am. J. Med.* **57**: 139-143) and experimental studies have revealed several ways in which excessive TNF- $\alpha$  production could promote cerebral malaria (Clark, I. A. (1987) *Parasitol. Today* **3**: 300-305; Grau, G. E. et al. (1987) *Science* **237**: 1210-1212).

In humans the gene for TNF- $\alpha$  resides within the class III region of the major histocompatibility complex (MHC) and several studies have shown that

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individual variation in the level of TNF- $\alpha$  production can be linked to HLA type and to other polymorphic markers in the MHC class III region (Molting, J. et al. (1988) *Scand. J. Immunol.* **27**: 705-716; Jacob, C.O. et al. (1990) *Proc. Natl. Acad. Sci. USA* **87**: 1233-1237; Pociot, F. et al. (1993) *Eur. J. Immunol.* **23**: 224-231). These observations suggest that TNF- $\alpha$  responsiveness may be controlled by variable genetic elements in the MHC region but the precise location of these elements has yet to be identified.

In the search for genetic elements involved in the regulation of TNF- $\alpha$  gene expression there has been much interest in a group of single nucleotide polymorphisms located at positions -238, -308 and -376 relative to the TNF- $\alpha$  transcriptional start site. All three polymorphisms are substitutions of adenine for guanine, the allelic types being hereinafter denoted TNF<sub>-238G/-238A</sub>, TNF<sub>-308G/-308A</sub> and TNF<sub>-376G/-376A</sub> respectively.

Population studies have shown that these polymorphisms in the TNF- $\alpha$  promoter may be associated with susceptibility to disease. For example, a study carried out in a population of Gambian children provided evidence that homozygotes for the TNF<sub>-308A</sub> allele have an increased risk of developing cerebral malaria (McGuire, W. et al. (1994) *Nature* **371**: 508-511). The TNF<sub>-308A</sub> allele has also been associated with susceptibility to a variety of other infectious diseases and chronic inflammatory diseases (Cabrera, M. et al. (1995) *J. Exp. Med.* **182**: 1259-1264; Conway, D.J. et al. (1997) *Infect. Immun.* **65**: 1003-1006; Nadel, S. et al. (1996) *J. Infect. Dis.* **174**: 878-880; Roy, S. et al. (1997) *J. Infect. Dis.* **176**: 530-532; Wilson, A. G. et al. (1994) *Eur. J. Immunol.* **24**: 191-195; Cox, A. et al. (1994) *Diabetologia* **37**: 500-503 and Louis, E. et al. (1996) *Gut* **39**: 705-710). However, at this time

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it remains an open question as to whether the disease associations with polymorphisms in the TNF- $\alpha$  promoter region are due to a direct effect on the TNF- $\alpha$  gene regulation, as opposed to linkage disequilibrium with functional polymorphisms elsewhere in the TNF- $\alpha$  locus or neighbouring genes.

The present inventors have surprisingly found that one of the known polymorphisms in the TNF- $\alpha$  promoter region is located within a site of DNA/protein recognition and thus has a direct functional effect on TNF- $\alpha$  transcription via altered transcription factor recruitment. The present inventors have further identified two novel DNA binding proteins which specifically recognise DNA sites in the region of the TNF- $\alpha$  promoter surrounding this polymorphism.

Accordingly, in a first aspect the invention provides a sequence-specific DNA binding protein which is capable of binding specifically to a DNA molecule having the sequence of nucleotides illustrated in SEQ ID NO: 1 or SEQ ID NO: 2.

Most preferably this protein, hereinafter referred to as the 21kDa protein, has an electrophoretic mobility equivalent to a protein of molecular weight 21 +/-5kDa when run on an SDS PAGE denaturing gel.

In a second aspect the invention provides a sequence-specific DNA binding protein which is capable of binding specifically to a DNA molecule having the sequence of nucleotides illustrated in SEQ ID NO: 3.

Most preferably this protein, referred to hereinafter as the 30kDa protein, has an electrophoretic mobility equivalent to a protein of molecular weight 30kDa +/- 5kDa when run on an SDS PAGE denaturing gel.

The invention also provides isolated nucleic acids having the nucleotide sequences illustrated in

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SEQ ID NO: 1 or SEQ ID NO: 2 or fragments thereof which are capable of specifically binding the above-described 21kDa DNA binding protein.

5 The invention also provides an isolated nucleic acid having the nucleotide sequence illustrated in SEQ ID NO: 3 a fragment thereof which is capable of binding specifically to the above-described 30kDa DNA binding protein.

10 The proteins of the invention are DNA binding proteins which have been characterised as binding specifically to DNA sequences within the promoter region of the TNF- $\alpha$  gene. As will be described in Example 2, the binding sites for these proteins were identified on the basis of solid phase DNAase I  
15 footprinting analysis of the region of the TNF- $\alpha$  promoter spanning positions -682 to -183 relative to the transcription start site using nuclear extracts from the cell line MonoMac6 which phenotypically resembles a well-differentiated human monocyte  
20 (Ziegler-Heitbrock, H.W.L. et al (1988) *Intl. J. Cancer* **41**: 456-461). The precise position of the protein binding sites has been further elucidated using electrophoretic mobility shift assays (EMSA) as will be described in detail in Example 3 below. Thus,  
25 the novel 21kDa DNA binding protein has been shown to bind to a site in the region of the TNF- $\alpha$  promoter extending from position -391 to position -374 relative to the transcription start site, having the nucleotide sequence set forth in Figure 1a. This region, which  
30 includes the previously identified -376 G/A polymorphism, is hereinafter referred to as the  $\alpha$  site. The novel 30kDa DNA binding protein has been shown to bind to a site in the region of the TNF- $\alpha$  promoter extending from position -365 to position  
35 -352 relative to the transcription start site, having the nucleotide sequence set forth in Figure 2. This region is hereinafter referred to as the  $\beta$  site.

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Complexes of the proteins of the invention bound to synthetic oligonucleotides comprising the sequence of the  $\alpha$  and  $\beta$  sites have been isolated using UV-crosslinking experiments and the molecular weight of the proteins has been determined using SDS-PAGE, as will be described in detail in Example 4.

The sequence-specific DNA binding properties of the 21kDa and 30kDa proteins can be readily exploited to purify the proteins on the basis of their binding specificity using techniques already known to those skilled in the art of protein purification. For example, the proteins may be purified from crude cell extracts (such as the nuclear extract of MonoMac6 cells or U937 cells described herein) on the basis of binding affinity by passing the nuclear extract over a solid support, such as a column matrix, to which have been attached synthetic oligonucleotides comprising a sequence corresponding to site  $\alpha$  or site  $\beta$ . The desired proteins, bound to the matrix, may then be released by using appropriate buffers, known in the art, and further purified by biochemical procedures such as, for example, SDS-PAGE electrophoresis or Reverse-Phase High Pressure Liquid Chromatography (RP-HPLC).

Partial amino acid sequences of the purified proteins may be determined using methods known to those skilled in the art of protein sequencing. For example, the purified molecule may be subjected to an Edman degradation reaction or, alternatively, to tandem mass-spectrometry. Prior to undertaking these procedures, it may be advantageous to cleave the purified molecule into smaller peptide fragments, for example, by using a protein cleaving enzyme such as trypsin followed by separation of the different fragments by Reverse-Phase High Pressure Liquid Chromatography (RP-HPLC).

Thus, in a still further aspect the invention

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provides a sequence-specific DNA binding protein which is obtainable by

- (a) preparing a crude nuclear extract from the human monocyte cell line U937;
- 5 (b) applying the nuclear extract of part (a) to a column of heparin sepharose CL-6B or phosphocellulose P11;
- (c) applying a salt gradient from 100 to 2000mM to the column;
- 10 (d) collecting a fraction of the eluate which is enriched for the said sequence-specific DNA binding protein;
- (e) incubating the enriched material obtained in step (d) with double-stranded DNA affinity probes
- 15 comprising the following sequence:

5'-GTTCTATCTTTTCTGCATCCTGTCTGGAAGTTA  
CAAGATAGAAAAAGGACGTAGGACAGACCTTCAAT-5'

- 20 to allow the formation of complexes of the sequence-specific DNA binding protein bound to the DNA affinity probe; and

- (f) recovering the sequence-specific binding protein from the complexes formed in step (e). The
- 25 material eluted from the column by applying the salt gradient in step (c) may be divided into fractions and tested, for example using EMSA, in order to identify fraction(s) which are enriched for the 21kDa DNA binding protein. This material may then be used in
- 30 step (e).

Also provided is a sequence-specific DNA binding protein which is obtainable by:

- (a) preparing a crude nuclear extract from the human monocyte cell line U937;
- 35 (b) applying the nuclear extract of part (a) to a column of heparin sepharose CL-6B or phosphocellulose P11;



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(c) applying a salt gradient from 100 to 2000mM to the column;

(d) collecting the material eluted at 250-350mM salt for heparin sepharose or 500-600mM salt for P11 phosphocellulose;

(e) incubating the enriched material obtained in step (d) with double-stranded DNA affinity probes comprising the following sequence:

10           5'-TAGAAGGAAACAGACCACAGACCTG  
              ATCTTCCTTTGTCTGGTGTCTGGAC-5'

to allow the formation of complexes of the sequence-specific DNA binding protein bound to the DNA affinity probe; and

(f) recovering the sequence-specific binding protein from the complexes formed in step (e).

The invention further provides oligonucleotide probes which are suitable for use in purifying the 21kDa and 30kDa DNA binding proteins on the basis of specific DNA-protein affinity binding. Thus the invention provides a nucleic acid molecule having the sequence of nucleotides illustrated in SEQ ID NO: 4 and a nucleic acid molecule having the sequence of nucleotides illustrated in SEQ ID NO: 5. These nucleic acid molecules contain, respectively, specific binding sites for the 21kDa DNA binding protein and the 30kDa DNA binding protein.

The DNA molecules of the invention are preferably double stranded and may be modified, for example by the addition of isotopic or non-isotopic labels. Also provided by the invention are materials comprising the above-described DNA molecules linked to a solid matrix or support. The solid matrix or support can be made of a wide variety of materials in a wide variety of shapes, including for example microbeads and resin particles which may be packed into a chromatographic

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column. Such material is particularly useful in the purification of the 21kDa or 30kDa DNA binding proteins by DNA affinity binding. The material is first incubated with a mixture containing the DNA binding protein, such as a crude or semi-purified nuclear extract, under conditions which allow for specific DNA-protein binding. The use of a solid matrix or support then facilitates the separation of the desired DNA-protein complexes from unbound components of the crude mixture. In a preferred embodiment, exemplified herein, the DNA molecule is labelled with biotin and is attached to solid microbeads (e.g. magnetic Dynabeads™) via a biotin-streptavidin binding interaction.

The two regions of the TNF- $\alpha$  promoter denoted herein site  $\alpha$  and site  $\beta$  have been identified as protein binding sites on the basis of DNAase I footprinting and EMSA experiments and are therefore putative *cis*-acting regulatory elements within the TNF- $\alpha$  promoter. Of the three previously described polymorphisms in the TNF- $\alpha$  promoter only one, the -376 G/A polymorphism, occurs within one of the putative *cis*-acting regulatory elements. As will be described in detail in Example 5, the present inventors were able to demonstrate that the -376 G/A substitution has a direct effect on TNF- $\alpha$  transcription by placing a 1.2kb fragment of the human TNF- $\alpha$  promoter upstream of a luciferase reporter gene and transiently expressing the construct in MonoMac6 cells. The -376 G/A polymorphism was introduced into the reporter gene construct by site-directed mutagenesis. Constructs containing the A substitution (-376A) showed a 35% increase in basal reporter gene expression compared to the wild-type promoter (-376G).

The present inventors have further demonstrated that the pattern of protein binding to site  $\alpha$  is markedly altered by the naturally occurring

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polymorphism at position -376. As will be described in detail in Examples 3 and 4, both EMA and UV-crosslinking experiments demonstrated that whilst the 21kDa DNA binding protein is able to bind to the  $\alpha$  site of both the TNF<sub>-376A</sub> and TNF<sub>-376G</sub> alleles, in the presence of the TNF<sub>-376A</sub> allele but not the TNF<sub>-376G</sub> allele a second protein of molecular weight of 95kDa (hereinafter referred to as the 95kDa protein) can bind to the  $\alpha$  site in addition to the 21kDa protein. Thus, sites  $\alpha$  and  $\beta$  of the TNF- $\alpha$  promoter normally interact with the 21kDa and 30kDa proteins respectively and the TNF<sub>-376A</sub> allele serves to recruit a third protein of about 95 kDa to the  $\alpha$  site. The present inventors have further demonstrated with the use of EMSA supershift and competition experiments that the 95kDa protein is the ubiquitously expressed transcription factor Oct-1 (Fletcher et al., Cell, 51: 773-781, 1987; Herr et al., Genes Dev, 9: 1679, 1995).

Knowledge of the specific DNA-protein binding interactions important in the regulation of TNF- $\alpha$  transcription can be used to design assays to identify compounds capable of disrupting the DNA-protein binding interactions and thus modulating TNF- $\alpha$  transcription. Compounds thus identified may be useful therapeutically in clinical situations in which it is desirable to up-regulate or down-regulate the level of TNF- $\alpha$  expression.

Accordingly, in a further aspect the invention provides a method of identifying compounds capable of modulating TNF- $\alpha$  gene expression, which method comprises the steps of:

- (a) contacting an aqueous solution comprising the 21kDa binding protein with a sample of the compound to form a reaction mixture;
- (b) contacting the reaction mixture of part (a) with a DNA fragment comprising the nucleotide sequence illustrated in SEQ ID NO: 1 or SEQ ID NO: 2;

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(c) observing the presence or absence of complexes comprising said DNA binding protein bound to said DNA fragment.

5 In a further aspect the invention provides a method of identifying compounds capable of modulating TNF- $\alpha$  gene expression, which method comprises steps of:

10 (a) contacting an aqueous solution comprising the 21kDa DNA binding protein and the transcription factor protein Oct-1 with a sample of the compound to form a reaction mixture;

(b) contacting the reaction mixture of part (a) with a DNA fragment comprising the sequence of nucleotides illustrated in SEQ ID NO: 2;

15 (c) observing the presence or absence of complexes comprising said DNA fragment.

The invention further provides a method of identifying compounds capable of modulating TNF- $\alpha$  gene expression, which method comprises steps of:

20 (a) contacting an aqueous solution comprising the 30kDa DNA binding protein with a sample of the compound to form a reaction mixture;

(b) contacting the reaction mixture of part (a) with a DNA fragment comprising the sequence of nucleotides illustrated in SEQ ID NO: 3.

25 (c) observing the presence or absence of complexes comprising said DNA binding protein bound to said DNA fragment.

30 The above methods of the invention can advantageously be performed in the form of an electrophoretic mobility shift assay (EMSA), as described in Example 3 below. A sample of the compound to be tested may be added to the reaction mixture prior to the addition of radiolabelled probe.

35 Binding reactions with and without the test compound are then analysed by electrophoresis. Compounds capable of disrupting the specific DNA-protein binding

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interaction will result in an absence of the band corresponding to the complex in a similar manner to the competition experiments described in Example 3. The steps of 'observing the presence or absence of complexes' might include simple qualitative detection of such complexes or quantitative measurement.

The invention also provides a method of identifying compounds capable of modulating TNF- $\alpha$  gene expression, which method comprises steps of:

10 contacting a DNA-protein complex comprising one of the following DNA/protein combinations:

- (i) a DNA fragment comprising the sequence of nucleotides illustrated in SEQ ID NO: 1 or SEQ ID NO: 2 and the 21kDa DNA binding protein,
- 15 (ii) a DNA fragment comprising the sequence of nucleotides illustrated in SEQ ID NO: 3 and the 30kDa DNA binding protein,
- (iii) a DNA fragment comprising the sequence of nucleotides illustrated in SEQ ID NO: 2, the 21kDa DNA binding protein and the transcription factor protein Oct-1,

20 with a sample of the compound and observing whether the DNA-protein complex is disrupted following contact with the compound.

25 In a further aspect the invention provides a method of identifying a compound capable of modulating TNF- $\alpha$  gene expression, which method comprises contacting a fragment of DNA comprising one or more of the sequences of nucleotides illustrated in SEQ ID NO: 1, SEQ ID NO: 2 or SEQ ID NO: 3 in the substantial absence of any other nucleotide sequences from the TNF- $\alpha$  promoter with a sample of the compound and detecting specific binding of the compound to the fragment of DNA.

35 The method of the invention is used to identify compounds capable of specifically binding to the  $\alpha$  and  $\beta$  sites within the TNF- $\alpha$  promoter, which compounds may

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thus be useful as modulators of the TNF- $\alpha$  transcription. Such compounds may be useful therapeutically in clinical situations in which it is desirable to up-regulate or down-regulate levels of TNF- $\alpha$ .

5 In order to isolate compounds which interact specifically the  $\alpha$  and/or the  $\beta$  site, the method of the invention uses  $\alpha$  and  $\beta$  site sequences isolated from the remainder of the TNF- $\alpha$  promoter, i.e. in the  
10 substantial absence of any other nucleotide sequences from the TNF- $\alpha$  promoter.

The readout of the assays of the invention may be provided by any technique known in the art to be useful in the detection of specific DNA/protein  
15 binding. Preferably, the DNA fragments of interest are labelled, either with biochemical markers or radioisotopes and binding of compounds is screened by techniques such as, for example, electrophoretic mobility shift assay (EMSA). Alternatively, specific  
20 binding interactions can be detected, without the need of labels, by proximity assay techniques such as, for example, flow cytometry or surface plasmon resonance. Advantageously, the assay method of the invention may be used to screen a combinatorial library of compounds  
25 in order to identify any compounds capable of specifically binding to site  $\alpha$  or site  $\beta$ .

In a still further aspect the invention provides a reporter gene expression construct comprising:

a reporter gene encoding a transcriptional and/or  
30 transnational product which can be directly or indirectly detected; and

a transcriptional control element comprising one or more of the sequences of nucleotides illustrated in SEQ ID NO: 1, SEQ ID NO: 2 or SEQ ID NO: 3 in the  
35 substantial absence of any other nucleotide sequence from a TNF- $\alpha$  promoter, the transcriptional control element being operably linked to the reporter gene.

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As used herein the term "operably linked" refers to a juxtaposition wherein the components described are in a relationship permitting them to function in their intended manner. In this case, the

5 transcriptional control element functions to drive transcription of the reporter gene.

Reporter genes suitable for use in the expression construct might include, for example, chloramphenicol acetyltransferase (CAT),  $\beta$ -galactosidase, firefly  
10 luciferase and green fluorescent protein. Following transfection into a host cell, reporter gene expression from the construct can be monitored either by direct measurement of mRNA or protein or by indirect measurement of properties of the protein such  
15 as enzymatic activity or fluorescence.

A suitable plasmid based reporter gene expression construct would include a reporter gene DNA with a downstream polyadenylation signal, an upstream  
20 multiple cloning site into which the transcriptional control element can be inserted, a synthetic polyadenylation signal upstream of the multiple cloning site to prevent read-through transcription from spurious promoter sequences in the vector backbone, a bacterial origin of replication and  
25 bacterial antibiotic resistance gene(s) to allow manipulation of the plasmid in a bacterial host strain. Suitable reporter gene expression vectors into which putative *cis*-acting regulatory elements of interest can be easily inserted are commercially  
30 available (for example from Promega, Madison, WI USA). The expression construct may advantageously contain further *cis*-acting promoter elements from a heterologous RNA polymerase II dependent promoter which function to increase the basal level of reporter  
35 gene expression.

The invention further provides cells which have been transfected with the expression constructs of the

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invention and still further provides a method of identifying compounds capable of modulating TNF- $\alpha$  gene expression, which method comprises comparing the amount of reporter gene expression in these cells in the presence of the compound with the amount of reporter gene expression in the absence of the compound or with the amount of reporter gene expression in cells transfected with a control reporter gene expression construct which does not contain the  $\alpha$  and  $\beta$  sites of the TNF- $\alpha$  promoter, whereby compounds capable of modulating TNF- $\alpha$  gene expression are identified.

In order to isolate compounds which interact specifically the  $\alpha$  and/or the  $\beta$  site, the reporter gene expression construct should preferably contain the  $\alpha$  and  $\beta$  site sequences isolated from the remainder of the TNF- $\alpha$  promoter, i.e. in the substantial absence of any other nucleotide sequences from the TNF- $\alpha$  promoter.

The method of the invention preferably uses cells which have been stably transfected to provide a cell line in which the reporter gene construct is stably integrated at a chromosomal location. To facilitate the production of stable cell lines one of the known eukaryotic selectable markers may be added to the reporter gene expression construct. Following transfection, stable lines may be selected and propagated by culturing in media containing the appropriate selective agent.

In contrast to the assay methods described above, which detect specific binding of compounds to the  $\alpha$  and  $\beta$  sites of the TNF- $\alpha$  promoter using physical techniques, the assay method based upon the use of cells transfected with reporter gene constructs containing the  $\alpha$  and  $\beta$  sites, uses a biological assay readout based on altered transcription. The assay may



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be used to identify compounds capable of modulating TNF- $\alpha$  expression which may be useful therapeutically in clinical situations in which it is desirable to up-regulate or down-regulate levels of TNF- $\alpha$ .

5           In a still further aspect the invention provides a method of screening human individuals for predisposition to inflammatory disease, which method comprises screening for the presence of a G to A transition polymorphism at position -376 in the  
10           promoter region of the TNF- $\alpha$  gene.

          Having identified that the -376 G/A polymorphism has a direct functional effect on TNF- $\alpha$  transcription *in vitro*, the present inventors performed a study to address the question of whether the -376 G/A  
15           polymorphism also exerts a functional effect on TNF- $\alpha$  regulation *in vivo* by examining the association between the polymorphism and susceptibility to cerebral malaria in two distinct African populations. As previously mentioned, clinical studies on cerebral  
20           malaria patients have demonstrated an association between host TNF- $\alpha$  levels and disease severity.

          In the first part of this study the inventors analysed DNA samples from a large case control study of severe malaria in Gambian children. As will be  
25           described in detail in Example 6 the Gambian study identified TNF<sub>-376A</sub> as an independent determinant of cerebral malaria with an estimated odds ratio of 4.3 when compared to a control group (95% confidence interval 1.5-12.8, P=0.008). On the basis of the  
30           Gambian study the inventors formulated the hypothesis that the TNF<sub>-376A</sub> allele is a determinant of susceptibility to cerebral malaria. In order to test this hypothesis the inventors examined the frequency of the TNF<sub>-376A</sub> allele in an independent case control  
35           study of severe malaria in Kenyan children. In the Kenyan study population the TNF<sub>-376</sub> allele was found to be in strong linkage disequilibrium with the TNF<sub>-238A</sub>

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allele ( $\Delta = 8.35$   $P < 0.001$ ). Logistic regression analysis of the two linked polymorphisms, combined with ethnic group, indicated that possession of the TNF-<sub>376A</sub> allele was associated with increased susceptibility to cerebral malaria (cerebral malaria cases vs controls: OR 5.0, 95% CI 1.5-17.0,  $P = 0.010$ ) while possession of the TNF-<sub>238A</sub> allele was associated with protection (OR 0.2, 95% CI 0.08-0.7,  $P = 0.008$ ). The present inventors have thus identified a strong relationship between the TNF-<sub>376A</sub> allele and susceptibility to cerebral malaria in two populations with widely divergent genetic features living on opposite sites of the African continent.

Cerebral malaria is just one example of an inflammatory disease in which TNF- $\alpha$  is known to play an important role. Given that the -376 G/A polymorphism has been shown by the present inventors to have a direct functional effect on the basal level of TNF- $\alpha$  transcription there is a reasonable expectation that the TNF-<sub>376A</sub> allele is also associated with susceptibility to other inflammatory diseases in which TNF- $\alpha$  is known to be an important factor such as, for example, rheumatoid arthritis, inflammatory bowel disease, systemic lupus erythematosus and the like. Accordingly, the method of the invention may be used to screen for susceptibility to any inflammatory disease in which TNF- $\alpha$  is known to be important. The method of the invention is particularly useful in screening for susceptibility to cerebral malaria.

The method of the invention involves determining the genotype of an individual at the -376 position of the TNF- $\alpha$  promoter (also referred to herein as 'genotyping') to establish whether the individual carries TNF-<sub>376A</sub> or TNF-<sub>376G</sub> alleles. The method may comprise screening for the presence or absence in the genome of the subject of both the TNF-<sub>376A</sub> allele and the TNF-<sub>376G</sub> allele or may comprise screening for the

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presence or absence of either individual allele, it generally being possible to draw conclusions about the genotype of an individual at a biallelic polymorphic locus just by screening for one or other of the variant alleles.

Genotyping may be carried out according to any of the techniques known to those skilled in the art of genetic screening. For example, genotyping may be accomplished with the method used by the present inventors in Example 6 part (b), namely PCR ELISA on samples of genomic DNA using differential hybridisation with allele specific oligonucleotide probes. The following oligonucleotide sequences are preferred for use as allele specific probes:

5'-CTGTCTGGAAgTTAGAAGGA (TNF<sub>-376G</sub> allele, SEQ ID NO:6)  
5'-CTGTCTGGAAaTTAGAAGGA (TNF<sub>-376A</sub> allele, SEQ ID NO:7)

Alternative techniques which can be used to perform genotyping at the TNF<sub>-376</sub> locus in accordance with the invention might include mass spectrometry, particularly matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF-MS, see Roskey, M. T. et.al., 1996, PNAS USA, 93: 4724-4729), single nucleotide primer extension (Shumaker, J. M. et.al., 1996, Hum. Mutat., 7: 346-354; Pastinen, T. et.al., 1997, Genome Res., 7: 606-614) and DNA chips or microarrays (Underhill, P. A. et.al., 1996, PNAS USA, 93: 196-200; Gilles, P. N. et.al. Nat. Biotech., 1999, 17: 365-370). In addition to the above, SNPs are commonly scored using PCR-based techniques, such as PCR-SSP using allele-specific primers (described by Bunce, 1995). Techniques for the scoring of DNA polymorphisms are reviewed by Schafer, A. J. and Hawkins, J. R. in Nature Biotechnology, Vol 16, pp33-39 (1998).

As would be readily apparent to those skilled in

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the art, genotyping is generally carried out on genomic DNA prepared from a suitable tissue sample obtained from the subject under test. Most commonly, genomic DNA is prepared from a sample of whole blood, according to standard procedures which are well known in the art.

The present invention may be further understood with reference to the following Examples together with the accompanying Figures in which:

10

Figure 1 shows the nucleotide sequence of the TNF- $\alpha$  promoter from position -391 to position -374 relative to the transcription start site, corresponding to the  $\alpha$  site; 1a is the wild type TNF-<sub>376G</sub> allele, 1b is the polymorphic variant TNF-<sub>376A</sub> allele.

15

Figure 2 shows the nucleotide sequence of the TNF- $\alpha$  promoter from position -365 to position -352 relative to the transcription start site, corresponding to the  $\beta$  site.

20

Figure 3 shows the results of a solid phase DNAase I footprinting experiment using a radiolabelled probe spanning the region from position -682 to position -183 of the TNF- $\alpha$  promoter, in the absence of nuclear extract (lanes 2 and 5) or incubated with nuclear extracts prepared from unstimulated MonoMac6 cells (lane 3) or MonoMac6 cells stimulated with lipopolysaccharide (LPS) (lane 4). Lane 1 is the Maxam Gilbert sequencing ladder for the same region of the TNF- $\alpha$  promoter.

25

30

Figure 4 shows the probes used in an electrophoretic mobility shift assay (EMSA) investigating the precise localisation of the  $\alpha$  site in the TNF- $\alpha$  promoter region.  $\alpha_G$  and  $\alpha_A$  probes correspond to the nucleotide sequence extending from position -407 to position -373

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- 19 -

of the TNF- $\alpha$  promoter, with either a G or an A at position -376. Proximal  $\alpha_G$  and  $\alpha_A$  probes correspond to the nucleotide sequence extending from position -391 to position -373 of the TNF- $\alpha$  promoter, with  
5 either a G or an A at position -376. Distal  $\alpha$  probe corresponds to the sequence extending from position -407 to position -386 of the TNF- $\alpha$  promoter.

Figure 5 shows the results of an electrophoretic mobility shift assay (EMSA) investigating the precise  
10 localisation of the  $\alpha$  site in the TNF- $\alpha$  promoter region. Nuclear extracts from unstimulated MonoMac6 cells were incubated with a radiolabelled  $\alpha_G$  (lane 1),  $\alpha_A$  (lane 4), distal  $\alpha$  (lane 2), proximal  $\alpha_G$  (lane 3)  
15 or proximal  $\alpha_A$  (lane 5) probe.

Figure 6 shows an electrophoretic mobility shift assay (EMSA) investigating the binding of Oct-1 to the  $\alpha$  site in the presence of an A substitution at position  
20 -376. Nuclear extracts from unstimulated MonoMac6 cells were incubated with a radiolabelled  $\alpha_G$  (lanes 1 to 7) or  $\alpha_A$  (lanes 8 to 14) probe either alone (lanes 1 and 8) or in the presence of anti-Oct-1 antibody (lanes 2 and 9), anti-p50 antibody (lanes 3 and 10) or  
25 unlabelled competitor probes (lanes 4 to 8 and 11 to 14).

Figure 7 shows the probes used in an electrophoretic mobility shift assay (EMSA) investigating the precise  
30 localisation of the  $\beta$  site in the TNF- $\alpha$  promoter region.  $\beta$  corresponds to the sequence from nucleotide -372 to nucleotide -352 of the TNF- $\alpha$  promoter;  $\beta$ -1 corresponds to the sequence from nucleotide -365 to nucleotide -345 of the TNF- $\alpha$  promoter;  $\beta$ -2 corresponds  
35 to the sequence from nucleotide -359 to nucleotide -339 of the TNF- $\alpha$  promoter.

- 20 -

Figure 8 shows the results of electrophoretic mobility shift assay (EMSA) investigating the precise localisation of the  $\beta$  site in the TNF- $\alpha$  promoter region. Nuclear extracts from LPS stimulated (lane 3) or unstimulated (lane 2 and 4-14) MonoMac6 cells were incubated with a radiolabelled  $\beta$  (lane 2, 3 and 11),  $\beta$ -1 (lane 13) or  $\beta$ -2 (lane 14) probe. A competition assay was also performed with a radiolabelled  $\beta$  probe in the presence of a 10x molar excess of an unlabelled  $\beta$  (lane 4),  $\beta$ -1 (lane 8),  $\beta$ -2 (lane 10) or EGR site (F:agctAAATCCCCGCCCCGCGATGGA) (lane 6) probe or in the presence of a 100x molar excess of unlabelled  $\beta$  (lane 5),  $\beta$ -1 (lane 9),  $\beta$ -2 (lane 11) or EGR site (lane 7) probe.

Figure 9 shows the results of UV-crosslinking experiments investigating proteins binding at the  $\alpha$  and  $\beta$  sites of the TNF- $\alpha$  promoter. EMSA binding reactions were performed with radiolabelled double stranded oligonucleotide probes comprising the  $\alpha$  site (with G or A at position -376, for complex I and II respectively) or the  $\beta$  site (complex III), in which five dT nucleotides were substituted with BrdU (as shown in Figure 10 below), complexes were crosslinked by UV light, excised and resolved using a 4-12% gradient polyacrylamide gel, either (a) directly or (b) after immunoprecipitation with anti-Oct-1 or control antibody.

Figure 10 shows the nucleotide sequence of the probes used in the cross-linking experiment described in Example 4. 5BrdU is shown as a Q.

Figure 11 is an EMSA using the  $\beta$  site specific probe (see below) illustrating the enrichment of proteins binding to site  $\beta$  of the TNF- $\alpha$  promoter using a P11

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column: Lane 1 crude nuclear extract, lane 2 crude nuclear extract following incubation with P11, lane 3 low salt buffer wash of P11, lane 4 enriched material eluted from P11 with high salt buffer.

5

Figure 12 is an EMSA using the  $\beta$  site specific probe (see below) illustrating the purification of proteins which bind specifically to site  $\beta$  of the TNF- $\alpha$  promoter using DNA affinity purification: Lane 1  
10 heparin sepharose enriched material, lane 2 bead-DNA purified material.

#### Example 1 Preparation of Nuclear Extracts

15 MonoMac6 cells ( $10-20 \times 10^6$ ) maintained in culture as previously described (Ziegler-Heitbrock, H. W. L. et al. *Int. J. Cancer*. **41**: 456-461 (1988)) were either harvested unstimulated or stimulated with 100ng/ml lipopolysaccharide (LPS) for 1 hour and then  
20 harvested. Nuclear extracts were prepared according to the method of Schrediber, E. et al. *Nucleic Acids Res.* **17**: 6419 (1989).

#### Example 2 Solid Phase DNAase I Footprinting.

25 Experimental method

Radiolabelled probes spanning the TNF- $\alpha$  promoter region from position -682 to position -183 relative to the transcription start site were generated by PCR  
30 using antisense primer 5'-GTTGGGGACACACAAGCATC (end labelled with  $\gamma^{32}\text{P}$ -dATP using T4 polynucleotide kinase) and biotinylated primer 5'-GCATTATGAGTCTCCGGGTC using TNFwt (-1173)-pXP1 plasmid DNA as template (construction of this plasmid is described in Example  
35 5 part (A) below. The DNAase I footprinting procedure itself was carried out according to the technique

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described by Sandaltzopoulos and Becker (*Nucleic Acids Research*. 22: 1511-1515 (1994)). Briefly, the DNA-binding reactions comprised radiolabelled DNA probe (40,000 cpm) absorbed onto magnetic Dynabeads™ M-280-Streptavidin (DYNAL, Norway) in binding reaction buffer (12mM HEPES, pH 7.8, 80-100mM KCl, 1mM EDTA, 12% glycerol and poly (dI-dC)) either alone (naked DNA) or incubated with 20µg crude nuclear extract prepared from MonoMac6 cells according to Example 1. The products of the DNA-binding reactions were subjected to DNAase I digestion 0.12-0.25U for 30 seconds, washed and analysed on a 7% acrylamide 7M urea gel. Areas of protection were localised by comparasion with a Maxam-Gilbert sequencing ladder.

### Results

Figure 3 shows that two novel sites of DNA-protein interaction are seen with nuclear extracts from LPS stimulated and unstimulated MonoMac6 cells. The first, designated  $\alpha$ , is located between position -404 and position - 374 of the TNF- $\alpha$  promoter region. The second, designated  $\beta$ , is located between position -371 and position -352. A further region of protection at position -600 is seen with nuclear extracts from LPS stimulated cells only and corresponds to the previously known NF- $\kappa$ B site cluster.

### Example 3 Electrophoretic Mobility Shift Assay.

#### Experimental method

Double stranded oligonucleotide probes were annealed and radiolabelled with an equimolar amount of  $\alpha^{32}\text{P}$ -dCTP using DNA polymerase I. Binding reactions were assembled containing 1µg crude nuclear extract prepared according to Example 1 and 4000 cpm



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radiolabelled probe in 10mM HEPES (pH 7.8), 50mM KCl, 1mM EDTA, 1mM EGTA, 12.5% glycerol, 1 $\mu$ g poly(dI-dC) (Amersham Pharmacia Biotech) and incubated for 20 minutes at room temperature. The binding reactions were analysed by electrophoresis in a non-denaturing 5% polyacrylamide gel at 4°C in 0.5% TBE buffer. EMSA supershift analysis was performed by pre-incubation of the reaction mixture with an appropriate antiserum (Santa Cruz) at room temperature for 10 minutes prior to addition of the radiolabelled probe.

### Results

#### a) Precise localisation of $\alpha$ site

In order to identify the precise region of the TNF- $\alpha$  promoter corresponding to the  $\alpha$  site, and EMSA assay was carried out as described above, incubating the different probes shown in Figure 4 with nuclear extracts from unstimulated MonoMac cells. Figure 5 shows that a low molecular weight complex (complex I) was observed with the  $\alpha_G$  (lane 1) and  $\alpha_A$  (lane 4) probes, as well as with the proximal  $\alpha_G$  (lane 3) and  $\alpha_A$  (lane 5) probes. An additional high molecular weight complex (complex II) was observed only with the  $\alpha_A$  (lane 4) and proximal  $\alpha_A$  (lane 5) probes, indicating that the G/A polymorphism at position -376 has a direct effect on recruitment of DNA-binding proteins at the  $\alpha$  site. No complex was observed with the distal  $\alpha$  probe (lane 2), showing that the 5' region of  $\alpha_G$  or  $\alpha_A$  is not involved in any DNA-protein interaction. These data, combined with those obtained from the Footprinting experiment of Example 2, suggest that the  $\alpha$  site is localised between nucleotide -391 and nucleotide -374 of the TNF- $\alpha$  promoter.

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**b) Characterisation of complex II**

The nature of complex II was further investigated by an EMSA supershift and competition assay, the results of which are shown in Figure 6. Complex II but not complex I was neutralised by the presence of antibody to Oct-1 (lanes 2 and 9) whilst neither complex was affected by anti-p50 (lanes 3 and 10). Complex I was competed by 100x molar excess of probe  $\alpha_G$  (lane 4) or probe  $\alpha_A$  (data not shown), both shown in Figure 4, but not by 100x molar excess of an oligoduplex probe matching an Oct-1 binding site from the human histone 2b gene (F:agctTCGCTTATGCAAATAAGGTGA) (lanes 5 and 6) or a probe matching the EGR site (F:agctAAATCCCCGCCCCGCGATGGA) (lane 7). In contrast, complex II was competed by a molar excess of  $\alpha_A$  (lane 11) and the probe matching the Oct-1 (lanes 12 and 13) but not by the probe matching the EGR site (lane 14) or probe  $\alpha_G$  (data not shown). The EMSA supershift and competition data indicate that in the presence of TNF-<sub>376A</sub> allele but not the presence of the TNF-<sub>376G</sub> allele Oct-1 binds to the  $\alpha$  site in addition to the 21kDa protein to form complex II.

**c) Precise localisation of  $\beta$  site**

In order to identify the precise region of the TNF- $\alpha$  promoter corresponding to the  $\beta$  site, an EMSA assay was carried out as described above, utilising the probes shown in Figure 7. Figure 8 shows that formation of a complex (complex III) was observed with the  $\beta$  (lanes 2,3 and 12) and  $\beta$ -1 (lane 13) probes but not with the  $\beta$ -2 probe (lane 14). Furthermore, formation of complex III with the  $\beta$  probe was competed by a molar excess of the  $\beta$  (lanes 4 and 5) or the  $\beta$ -1 (lanes 8 and 9) probe but not by the

- 25 -

$\beta$ -2 probe (lanes 10 and 11) or by an irrelevant probe (lanes 6 and 7).

These results, combined with those obtained from the Footprinting experiment of Example 2, suggest that the  
5  $\beta$  site is localised between nucleotide -365 and -352 of the TNF- $\alpha$  promoter region.

#### Example 4 UV-crosslinking

10

##### Experimental methods

UV-crosslinking was carried out by first performing EMSA experiments using radiolabelled oligoduplex probes in which specified dT nucleotides were  
15 substituted with BrdU, as shown in Figure 10. The EMSA gel was then illuminated with UV radiation at 302nm for 30 minutes at 4°C and exposed to autoradiography film for four hours at the same temperature. The autoradiography film was used to  
20 locate regions of the EMSA gel corresponding to the specific protein-DNA complexes. The relevant fragments of gel were then excised and the complexes eluted in 2x SDS buffer (100mM Tris-Cl pH 6.8, 200mM DTT, 2% SDS, 20% glycerol) at 37°C overnight. The  
25 complexes thus isolated were then resolved using a 4-12% gradient polyacrylamide gel either directly or after immunoprecipitation as previously described (Hansen, S.K. et al. *EMBO J.* 11: 205-213).

##### Results

Figure 9 shows the results of UV-crosslinking experiments to investigate proteins binding at site  $\alpha$  and  $\beta$  of the TNF- $\alpha$  promoter using  $\alpha$  and  $\beta$  site  
35 oligoduplex probes. Figure 9(a) 4-12% gradient SDS-PAGE of complexes formed by incubation of crude

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nuclear extracts from unstimulated MonoMac6 cells with  $\alpha$  site probes containing A (lane 1) or G (lane 2) at position -376 or with  $\beta$  site probes (lane 3), all shown in Figure 10. The molecular weights of the complexes were determined by comparing their electrophoretic mobility with that of molecular weight markers. The molecular weights of the proteins binding to site  $\alpha$  or  $\beta$  were then calculated by subtracting the molecular weight of the oligonucleotide probes. Two proteins of about 21kDa and 30kDa were shown to bind to the  $\alpha$  and  $\beta$  sites, respectively. Furthermore, another protein of about 95kDa was shown to bind, together with the 21kDa protein, only to the  $\alpha_A$  site but not to the  $\alpha_G$  site. The estimates of molecular weight are accurate only to  $\pm 5$ kDa, since the migration of the complexes may be influenced by various structural variables. Figure 9(b) shows SDS-PAGE after immunoprecipitation of complexes I and II with anti-Oct-1 or a control unrelated antibody (anti-p50 NF- $\kappa$ B). UV crosslinked forms of complex II but not of complex I could be specifically immunoprecipitated by anti-Oct-1 antibodies, showing that the protein of about 95kDa is Oct-1. Both Figure 9(a) and 9(b) show that the binding of Oct-1 is specific for the TNF<sub>-376A</sub> allele.

### Example 5 Reporter gene analysis

#### (a) Plasmid construction

The plasmid TNFwt (-1173)-pXP1 was constructed by placing a ~1.2kb fragment of the wild type TNF- $\alpha$  promoter and 5' untranslated region from position -1173 to position +130 relative to the transcriptional start site upstream of a firefly luciferase reporter gene in the eukaryotic expression vector pXP1 (described by Nordeen S.K. (1988) *Biotechniques* 6: 454-456). The -1173 to +130 fragment of wild type

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TNF- $\alpha$  was obtained as a HindIII/NdeI fragment from the previously described plasmid -1173-CAT (Udalova I.A. et al. (1995) *Doklady Akademii Nauk.* **342**: 413-417).

5 The corresponding fragment containing the G to A substitution at position -376 was synthesised by site directed mutagenesis in the construct -1173-CAT, using previously published methodology (Stuber F. et al (1996) *J. Inflamm.* **46**: 42-50).

10 **(b) Transient transfection of MonoMac 6 cells**

Transfection of MonoMac 6 cells was carried out using the modified DEAE-dextran method (Shakhov, A et al. (1990) *J. Exp. Med.* **171**: 35-47) using 100 $\mu$ g/ml DEAE-dextran and 2 $\mu$ g plasmid DNA. The cells were allowed to recover for 24 hours post-transfection and then either left unstimulated or stimulated with 200ng/ml LPS. The cells were harvested after 40 hours with cell lysate volumes adjusted to equalise total  
15 cellular protein content between samples. Firefly luciferase activity was measured using a manual Turner TD20e luminometer (Promega).  
20

**Results**

25

The results of transient transfection experiments using MonoMac6 cells are summarised in Table 1 below:

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Table 1

	No Insert	376G	376A	P Value*
Basal	0.23±0.07	16.1±1.7	21.7±2.4	0.002
LPS- stimulated	0.25±0.1	189.9±19.3	200.3±13.7	ns
Ratio of LPS stimulated: basal	1.1±0.5	12.3±1.4	10.1±1.5	0.007

- 10 \*P values shown for comparison of 376G and 376A  
constructs by two-tailed paired t-test. MonoMac6 cells  
were transfected with pXP1 vector containing no  
insert, which served as a negative control, or with  
one of two allelic forms of the TNF- $\alpha$  promoter region  
15 which differed by a single nucleotide substitution at  
position -376, either G (denoted as 376G) or A  
(denoted as 376A). The mean of 7 independent  
transient transfection experiments is shown ( $\pm$ SEM)  
using two independent plasmid preparations. Cells were  
20 unstimulated to determine basal expression or  
stimulated with a high dose of LPS (200ng/ml).  
Luciferase activity of cell lysates was determined  
using a luminometer (with results corrected for total  
cell protein) and is expressed as luminometer units  
25 per mg protein.

#### Example 6 Clinical case-control studies.

##### (A) Study design

- 30 Children under 10 years of age were recruited to two  
independent case-control studies in the Gambia and  
Kenya. The Gambian study was carried out near Banjul  
and was hospital based as described previously (Hill,  
A.V.S. et al. (1991) *Nature* 352: 595-600). In the  
35 Kenyan study cases of severe malaria recruited at the

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Kilifi District Hospital were matched for age with community controls (Newbold, C. et al. (1997) *J. Trop. Med. Hygiene*. 57: 398; Snow, R.W. et al. (1993) *Trans. R. Soc. Trop. Med. Hyg.* 87: 386-390). The studies were approved by the MRC/Gambian joint ethical committee and the KEMRI ethical committee. Cerebral malaria was defined as a Blantyre coma score of 2 or less (Molyneux, M.E. et al. (1989) *Q. J. Med.* 71: 441-459) persisting for more than 30 minutes after any convulsions had ceased in a child with *P. falciparum* parasites on thick blood film and no evidence of meningitis or any other cause of coma. The Gambian control group comprised children seen in clinic with a variety of mild non-malarial illnesses that did not require admission to hospital. The Kenyan control group comprised healthy age-matched children from the same community as the index cases, recruited irrespective of presence or absence of malarial parasitaemia. The mean age of children in the Gambian study was 3.9 years for cerebral malaria cases and 2.9 years for the controls, whilst in the Kenyan study it was 2.7 years and 2.5 years respectively. Data was also analysed from Gambian children with severe malarial anaemia (haemoglobin <5g/dl) or mild malaria (defined as an uncomplicated febrile illness in a child with asexual *P. falciparum* parasites on the blood film without any other satisfactory explanation for the fever).

30 (B) Genotyping of TNF<sub>376</sub> and TNF<sub>238</sub>

Genotyping was carried out on randomised samples of genomic DNA, blind to disease outcome, by PCR ELISA (Boehringer Mannheim) using differential hybridisation with sequence specific probes. Nested PCR amplification generated a digoxigenin labelled 375

- 30 -

nucleotide product spanning position -558 to position -183 of the TNF- $\alpha$  promoter using 100ng of genomic DNA as a template. This product was alkaline denatured and hybridised in streptavidin coated microtitre plates with allele-specific 5' biotinylated oligonucleotide probes at 55°C for 2 hours. Allele-specific capture probes used for TNF<sub>-238</sub> were 5'-CCTCGGAATCgGAGCAGGGA and 5'-CCTCGGAATCaGAGCAGGGA at 2.5 pmol/ml; for TNF<sub>-376</sub> 5'-CTGTCTGGAAgTTAGAAGGA and 5'-CTGTCTGGAAaTTAGAAGGA at 15 pmol/ml. A split plate design was used for each polymorphism, including positive and negative controls. Hybridised DNA was labelled by incubation with 2mU anti-digoxigenin peroxidase conjugate for 30 minutes at 37°C. After addition of 0.2mg of ABTS substrate plates were read on an ELISA plate reader at 405nm (reference filter 492nm). The accuracy of genotyping data obtained by PCR ELISA was confirmed by DNA sequencing.

## 20 Results

Results from the Gambian case control study are summarised in Table 2 below:

25 Table 2

	Cerebral malaria	Severe malaria anaemia	Mild malaria	Non- malaria controls
<b>TNF<sub>-376A</sub></b>				
<i>n</i>	384	132	349	371
Heterozygotes	24 (6.3%)	5 (3.8%)	13 (3.7%)	9 (2.4%)
Homozygotes	1 (0.3%)	0	1 (0.3%)	1 (0.3%)

*n* number of individuals in each clinical category for the Gambian case-control study. In a 2x2 analysis



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possession of the TNF-<sub>376A</sub> allele was associated with an odds ratio of 2.5 (95% CI 1.2-5.3)  $\chi^2=6.21$   $P=0.013$ ) for cerebral malaria versus non-malaria controls.

5     **Example 7 Affinity purification of proteins**

Proteins binding to the  $\alpha$  and  $\beta$  sites of the TNF- $\alpha$  promoter are present in crude nuclear extracts prepared from the human monocyte cell line U937  
10     (CellBank, Dunn School of Pathology, University of Oxford; refs International Journal of Cancer, 17, 565-577, (1976) and Journal of Immunology 125, 6-12 (1980)). This cell line is used as a starting material for purification of the 21kDa and 30kDa  
15     proteins in preference to MonoMac6 as it is more suitable for large scale cell culture work.

**1) Enrichment of specific proteins binding to sites  $\alpha$  and  $\beta$  using affinity chromatography**

20     Generic partial purification of DNA binding proteins has been reported using a variety of ion exchange and other resins, for example DNA-cellulose, phosphocellulose, DE52, wheat germ agglutinin and CMS2  
25     (see Methods in Enzymology 208, 10-23 (1991) for examples of proteins purified by DNA affinity). The inventors have found that proteins binding to site  $\alpha$  and site  $\beta$  can be enriched specifically by heparin sepharose and phosphocellulose (P11), using as a read  
30     out for successful binding an electrophoretic mobility shift assay (EMSA) using radiolabelled probes containing the specific DNA recognition sequences for the respective proteins. EMSA methodology was described previously in Example 3. Probe design also  
35     as documented previously:

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site  $\alpha$     5'agctGTTCTATCTTTTTCCTGCATCCTGTCTGGAAGTTA  
             5'agctTAACTTCCAGACAGGATGCAGGAAAAAGATAGAAC

5            site  $\beta$     5'agctTAGAAGGAAACAGACCACAGACCTG  
             5'agctCAGGTCTGTGGTCTGTTTCCTTCTA

Crude nuclear extract prepared from unstimulated U937 cells will bind to heparin sepharose CL-6B (Pharmacia) and to phosphocellulose (P11) (Whatman) using BC100  
10 buffer (20mM Hepes, 20% glycerol, 0.2mM EDTA and 100mM NaCl). The active material containing the protein(s) binding to site  $\beta$  are eluted by applying a salt gradient from 100 to 2000mM with enriched material eluted at 250-350mM (heparin sepharose) and 500-600mM  
15 (P11) BC buffer. Figure 11 illustrates this for P11 in an EMSA: lane 1 crude nuclear extract, lane 2 crude nuclear extract following incubation with P11 (demonstrating the protein is absent as it has bound to P11), lane 3 low salt buffer wash of P11  
20 (demonstrating the protein remains bound to P11), lane 4 enriched material eluted from P11 with high salt buffer.

## 2) DNA affinity purification

25 Following heparin sepharose/P11 purification, the proteins binding to sites  $\alpha$  and  $\beta$  are recovered by DNA affinity purification using site-specific oligonucleotide probes. The generic methodology for purification using biotinylated oligonucleotide probes  
30 linked to streptavidin coated beads has been described (Nucleic Acids Research 17, 6253-6267 (1989)). For site  $\beta$  the following biotinylated probe is synthesised:

35    5'agctTAGAAGGAAACAGACCACAGACCTG  
             ATCTTCCTTTGTCTGGTGTCTGGAC-biotin-5'

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For site  $\alpha$  the following biotinylated probe is synthesised:

5'agctGTTCTATCTTTTTCCTGCATCCTGTCTGGAAGTTA

5 CAAGATAGAAAAAGGACGTAGGACAGACCTTCAAT-biotin-5'

Procedures for the labelling of oligonucleotides with biotin are well known in the art.

The appropriate double-stranded probe is added to streptavidin-coated Dynabeads™ (M-280) to which the probe becomes strongly bound. Incubation of the probe-bead complex with the enriched material generated in part 1) allows binding of the proteins specific to the site of interest defined by the sequence of the oligonucleotide probe. Figure 12 shows the result of such a purification step for site  $\beta$  when the products are used in an EMSA (lane 1 heparin sepharose purified material, lane 2 bead-DNA purified material).

20

#### SEQUENCE LISTING

1. Sequence ID No. 1 corresponds to the nucleotide sequence of the  $\alpha$  site of the human TNF- $\alpha$  promoter illustrated in Figure 1a (TNF-<sub>376G</sub> allele).  
25
2. Sequence ID No. 2 corresponds to the nucleotide sequence of the  $\alpha$  site of the human TNF- $\alpha$  promoter illustrated in Figure 1b (TNF-<sub>376A</sub> allele).  
30
3. Sequence ID No. 3 corresponds to the nucleotide sequence of the  $\beta$  site of the human TNF- $\alpha$  promoter illustrated in Figure 2.  
35
4. Sequence ID No. 4 is the nucleotide sequence of

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an oligonucleotide probe corresponding to a region of the human TNF- $\alpha$  promoter including the  $\alpha$  site.

- 5      5.      Sequence ID No. 5 is the nucleotide sequence of an oligonucleotide probe corresponding to a region of the human TNF- $\alpha$  promoter including the  $\beta$  site.
- 10     6.      Sequence ID No. 6 is the nucleotide sequence of an allele-specific oligonucleotide probe specific for the TNF-<sub>376G</sub> allele.
- 15     7.      Sequence ID No. 7 is the nucleotide sequence of an allele-specific oligonucleotide probe specific for the TNF-<sub>376A</sub> allele.

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**Claims:**

1. A sequence-specific DNA binding protein which is capable of binding specifically to a DNA molecule having the sequence of nucleotides illustrated in SEQ ID NO: 1 or SEQ ID NO: 2.
2. A sequence-specific DNA binding protein as claimed in claim 1 having an electrophoretic mobility equivalent to a protein of molecular weight 21 +/- 5kDa when run on an SDS PAGE denaturing gel.
3. A sequence-specific DNA binding protein as claimed in claim 1 or claim 2 which is obtainable by
- (a) preparing a crude nuclear extract from the human monocyte cell line U937;
  - (b) applying the nuclear extract of part (a) to a column of heparin sepharose CL-6B or phosphocellulose P11;
  - (c) applying a salt gradient from 100 to 2000mM to the column;
  - (d) collecting a fraction of the eluate which is enriched for the said sequence-specific DNA binding protein;;
  - (e) incubating the enriched material obtained in step (d) with double-stranded DNA affinity probes comprising the following sequence:

5'-GTTCTATCTTTTCCTGCATCCTGTCTGGAAGTTA  
CAAGATAGAAAAAGGACGTAGGACAGACCTTCAAT-5'

to allow the formation of complexes of the sequence-specific DNA binding protein bound to the DNA affinity probe; and

- (f) recovering the sequence-specific binding protein from the complexes formed in step (e).

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4. A sequence-specific DNA binding protein which is capable of binding specifically to a DNA molecule having the sequence of nucleotides illustrated in SEQ ID NO: 3.

5

5. A sequence-specific DNA binding protein as claimed in claim 4 having an electrophoretic mobility equivalent to a protein of molecular weight 30 +/- 5kDa when run on an SDS PAGE denaturing gel.

10

6. A sequence-specific DNA binding protein as claimed in claim 4 or claim 5 which is obtainable by:

(a) preparing a crude nuclear extract from the human monocyte cell line U937;

15 (b) applying the nuclear extract of part (a) to a column of heparin sepharose CL-6B or phosphocellulose P11;

(c) applying a salt gradient from 100 to 2000mM to the column;

20 (d) collecting the material eluted at 250-350mM salt for heparin sepharose or 500-600mM salt for P11 phosphocellulose;

(e) incubating the enriched material obtained in step (d) with double-stranded DNA affinity probes  
25 comprising the following sequence:

5'-TAGAAGGAAACAGACCACAGACCTG  
ATCTTCCTTTGTCTGGTGTCTGGAC-5'

30 to allow the formation of complexes of the sequence-specific DNA binding protein bound to the DNA affinity probe; and

(f) recovering the sequence-specific binding protein from the complexes formed in step (e).

35

7. An isolated nucleic acid having the sequence of nucleotides illustrated in SEQ ID NO: 1.

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8. An isolated nucleic acid having the sequence of nucleotides illustrated in SEQ ID NO: 2.

5 9. An isolated nucleic acid as claimed in claim 7 or claim 8 or a fragment thereof which is capable of specifically binding to the DNA binding protein of any one of claims 1 to 3.

10 10. An isolated nucleic acid having the sequence of nucleotides illustrated in SEQ ID NO: 3.

15 11. An isolated nucleic acid as claimed in claim 10 or a fragment thereof which is capable of specifically binding to the DNA binding protein of any one of claims 4 to 6.

12. A reporter gene expression construct comprising:

20 a reporter gene encoding a transcriptional and/or translational product which can be directly or indirectly detected; and

25 a transcriptional control element comprising one or more of the sequences of nucleotides illustrated in SEQ ID NO: 1, SEQ ID NO: 2 or SEQ ID NO: 3 in the substantial absence of any other nucleotide sequence from the TNF- $\alpha$  promoter, the transcriptional control element being operably linked to the reporter gene.

30 13. A reporter gene expression construct as claimed in claim 12 which further comprises one or more *cis*-acting promoter or enhancer elements from a heterologous promoter.

35 14. Cells transformed or transfected with the reporter gene expression construct of claim 12 or claim 13.

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15. A method of identifying a compound capable of modulating TNF- $\alpha$  gene expression, which method comprises:

5       contacting a fragment of DNA comprising one or more of the sequences of nucleotides illustrated in SEQ ID NO: 1, SEQ ID NO: 2 or SEQ ID NO: 3 in the substantial absence of any other nucleotide sequences from a TNF- $\alpha$  promoter with a sample of the compound and detecting any specific binding of the compound to  
10       the fragment of DNA.

16. A method of identifying compounds capable of modulating TNF- $\alpha$  gene expression, which method comprises:

15       comparing the difference in the amount of reporter gene expression in the cells of claim 14 in the presence of the compound with the amount of reporter gene expression in the absence of the compound and/or with the amount of reporter gene  
20       expression in cells transfected with a control reporter gene expression construct which does not contain one or more of the sequences of nucleotides illustrated in SEQ ID NO: 1, SEQ ID NO: 2 or SEQ ID NO: 3, whereby compounds capable of modulating TNF- $\alpha$   
25       gene expression are identified.

17. A method of identifying compounds capable of modulating TNF- $\alpha$  gene expression, which method comprises steps of:

30       (a) contacting an aqueous solution comprising a DNA binding protein as claimed in any one of claims 1 to 3 with a sample of the compound to form a reaction mixture;

35       (b) contacting the reaction mixture from part (a) with a DNA fragment comprising the sequence of nucleotides illustrated in SEQ ID NO: 1 or SEQ ID NO: 2; and



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(c) observing the presence or absence of complexes comprising said DNA binding protein bound to said DNA fragment.

5           18. A method of identifying compounds capable of modulating TNF- $\alpha$  gene expression, which method comprises steps of:

(a) contacting an aqueous solution comprising the DNA binding protein claimed in any one of claims 1  
10 to 3 and the transcription factor protein Oct-1 with a sample of the compound to form a reaction mixture ;

(b) contacting the reaction mixture of part (a) with a DNA fragment comprising the sequence of nucleotides illustrated in SEQ ID NO: 2; and

15           (c) observing the presence or absence of complexes comprising said DNA binding protein bound to said DNA fragment.

20           19. A method of identifying compounds capable of modulating TNF- $\alpha$  gene expression, which method comprises steps of:

contacting an aqueous solution comprising the DNA binding protein claimed in any one of claims 4 to 6 with a sample of the compound to form a reaction  
25 mixture;

(b) contacting the reaction mixture of part (a) with a DNA fragment comprising the sequence of nucleotides illustrated in SEQ ID NO: 3; and

30           (c) observing the presence or absence of complexes comprising said DNA binding protein bound to said DNA fragment.

20. A method as claimed in any one of claims 17 to 19 wherein said DNA fragment is radiolabelled and  
35 the presence or absence of complexes comprising said DNA binding protein bound to said DNA fragment is determined by electrophoretic mobility shift assay.

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21. A method of identifying compounds capable of modulating TNF- $\alpha$  gene expression, which method comprises:

- 5       contacting a DNA-protein complex comprising one of the following DNA/protein combinations:
- (i) a DNA fragment comprising the sequence of nucleotides illustrated in SEQ ID NO: 1 or SEQ ID NO: 2 and the DNA binding protein claimed in any one of claims 1 to 3,
  - 10   (ii) a DNA fragment comprising the sequence of nucleotides illustrated in SEQ ID NO: 3 and the DNA binding protein claimed in any one of claims 4 to 6,
  - 15   (ii) a DNA fragment comprising the sequence of nucleotides illustrated in SEQ ID NO: 2, the DNA binding protein claimed in any one of claims 1 to 3 and the transcription factor protein Oct-1,
  - 20   with a sample of the compound and observing whether the DNA-protein complex is disrupted following contact with the compound.

22. A compound capable of modulating TNF- $\alpha$  gene expression, which compound has been identified using the method of any one of claims 15 to 21.

25

23. A nucleic acid molecule having the sequence of nucleotides illustrated in SEQ ID NO: 4.

24. A nucleic acid molecule as claimed in claim 23 which is a double-stranded DNA molecule.

30

25. A material comprising the nucleic acid molecule of claim 23 or claim 24 attached to a solid matrix or support.

35

26. A material as claimed in claim 25 wherein the nucleic acid molecule is labelled with biotin and

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is attached to the solid support or matrix via a biotin/streptavidin binding interaction.

27. Use of the material claimed in claim 25 or  
5 claim 26 in a procedure for purifying a sequence-specific DNA binding protein as defined in any one of claims 1 to 3.

28. A nucleic acid molecule having the sequence  
10 of nucleotides illustrated in SEQ ID NO: 5.

29. A nucleic acid molecule as claimed in claim 28 which is a double-stranded DNA molecule.

15 30. A material comprising the nucleic acid molecule of claim 28 or claim 29 attached to a solid matrix or support.

20 31. A material as claimed in claim 30 wherein the nucleic acid molecule is labelled with biotin and is attached to the solid support or matrix via a biotin/streptavidin binding interaction.

25 32. Use of the material claimed in claim 30 or claim 31 in a procedure for purifying a sequence-specific DNA binding protein as defined in any one of claims 4 to 6.

30 33. A method of screening human individuals for predisposition to inflammatory disease, which method comprised screening for the presence of a G to A transition polymorphism at position -376 in the promoter region of the TNF- $\alpha$  gene.

35 34. A method as claimed in claim 33 wherein the inflammatory disease is cerebral malaria.

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35. A method as claimed in claim 33 or claim 34 wherein said screening for the presence of a G to A transition polymorphism at position -376 in the promoter region of the TNF- $\alpha$  gene is carried out using  
5 PCR ELISA.

36. A method as claimed in claim 35 wherein said PCR ELISA is carried out using allele-specific  
oligonucleotide probes having the following sequences:  
10 5'-CTGTCTGGAAGTTAGAAGGA (SEQ ID NO: 6)  
5'-CTGTCTGGAAATTAGAAGGA (SEQ ID NO: 7)